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[54] 发明名称 用于重组腺伴随病毒生产的全功能辅助病毒的产生及其用途

[57] 摘要

本发明包括了一种装载了2型腺伴随病毒(AAV—2)rep—cap基因的重组单纯疱疹病毒(HSV1—rc)的产生方法及其在重组腺伴随病毒(rAAV)生产中的用途。这种重组单纯疱疹病毒能提供rAAV质粒在细胞内复制和包装成rAAV毒粒所需的全部辅助功能，并能用于rAAV的大量制备。HSV1—rc的产生是在对一套含有HSV1病毒全基因组的粘性质粒(Set C粘粒，包括cos6，cos14，cos28，cos48，cos56)进行改造的基础上实现的。首先，用重组DNA技术将AAV—2rep—cap基因插入其中一个粘粒的HSV—1基因组中，例如插入cos6的HSV1 UL2基因中构建成cos6—rcΔUL2；插入cos56的HSV1 UL44基因中构建成cos56—rcΔUL44。然后，将插入了rep—cap的重组粘粒与相应的其余4个粘粒经酶切切去粘粒骨架部分后用脂质体方法共转染HSV1敏感细胞如BHK—21，5个HSV1片段在细胞中发生同源重组而产生HSV1—rc。用HSV1—rc感染rAAV载体质粒转染的细胞或稳定携带rAAV载体质粒的细胞株，就能产生大量有感染性的rAAV毒粒。用这种方法产生的rAAV能将外源基因导入哺乳动物细胞中并表达。

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Process for preparing of full-function helper virus used for producing recombinant adeno-associated virus and uses thereof

The present invention relates to the field of genetic engineering in virus, particularly to the replicating and packaging function system that are necessary for large production of recombinant adeno-associated virus (rAAV). The invention relates to a method of producing recombinant herpes simplex virus (HSV1-rc) that is loaded with rep-cap genes (403kb) of type2 adeno-associated virus (AAV-2) and the use thereof in the production of recombinant adeno-associated virus. The recombinant herpes simplex virus can provide the complete helper functions that are necessary for rAAV plasmid to be replicated and packaged in cells to be rAAV virus particles, and can be used in large preparation of rAAV. The production of HSV1-rc is carried out based on the modification for a set of cosmids (Set C cosmid, including cos6, cos14, cos28, cos48, cos56) that contain the full-length genome of rAAV virus particle. Lots of infectious rAAV virus particles can be produced by using HSV1–rc to infect the cells transfected with rAAV vector plasmid or the cell strain that carry stably rAAV vector plasmid. Exogenous genes can be introduced into mammalian cells for expression by using rAAV thus produced.

Adeno-associated virus is a member of parvovirus, with a single-stranded DNA genome consists of 4682nt. AAV is a dependent virus of which the replication needs the participation of other viruses such as adenovirus or herpes simplex virus, or other helper factors. The genome of AAV will be integrated into the chromosome of cells in latency upon AAV infection in the absence of helper virus, without generating progeny viruses.

The full-length genome of AAV-2 has been cloned into the plasmid of E. coli. Two 145bp length of inverted terminal repeat sequences (ITR) are contained in the genome. The two ITRs are replication origins of AAV genome and participate in such functions as replication, integration, or package of AAV, etc.. The rest parts of the genome are divided into two functional regions, rep gene region and cap gene region. The rep gene encodes four different types of products: Rep78, Rep68, Rep52, Rep40, which are regulatory proteins necessary for the replication and gene expression of AAV. The cap gene encodes three types of structural proteins: VP1, VP2, VP3 which are assembled together into the capsid of AAV . The proteins encoded by rep and cap genes are trans-acting proteins in the toxicogenic replication of AAV.

AAV is considered as one of the ideal candidate vectors in gene therapy. A rAAV virus into which exogenous genes can be transduced has been constructed in a lot of laboratories. The main structural characteristic of AAV vector plasmid is the remove of rep-cap genes from the genome of the virus and the substitution of the desired DNA fragment.

The classical method of producing rAAV is transducing rAAV vector plasmid and a helper vector that contains rep-cap genes into the cells that has been infected by adenovirus or herpes simplex virus. 2-3 days later, rAAV as well as adenoviruses and herpes simplex viruses, can be harvested from the supernatant and the pathological cells. Adenoviruses and herpes simplex viruses can be inactivated by

heat treatment(at 55℃ for 30min to 2hr) without affecting the activities of AAV.

Although the method of producing rAAV is simple, it still exists a lot of disadvantages. Firstly, cells need to be transfected in each preparation of rAAV. One of the factors that result in lower titer of rAAV is the low efficiency of transfection and co-transfection due to the restriction of the transfection method itself. Furthermore, it is difficult for cells to be transduced on a large scale by transfection method by now, accordingly it can't meet the needs of producing rAAV largely. Thus a system and method that can be used to produce rAAV largely needs to be studied.

Yan ziying et al have ever filed a patent application named "A herpes simplex virus vector that can be used to pack adeno-associated virus and the use thereof" (Chinese patent application number 96120549.0, publication number CN1159480A) in 1996. The application described that the rep-cap genes of AAV-2 are located in the amplicon vector plasmid of HSV1 to construct pHSV–AAV (+/-). The plasmid was introduced into cells, and a mixed virus of a wild-type HSV-1 and a pseudovirus that contained rep-cap genes were obtained in the presence of the wild-type HSV-1, wherein the mixed virus can provide the complete helper function for the replication and package of rAAV. Recently, Conway et al (Conway JE et al, Recombinant adeno-associated virus type 2 replication and packaging is entirely supported by a herpes simplex virus type 1 amplicon expressing rep and cap J. Virol. 71(11): 8780-8789, 1997) also reported the similar studies. However the pseudovirus in the mixed virus occupied a small proportion (<10%), and provided limited helper function; furthermore the proportion between the pseudovirus and the wild-type virus is unstable in virus passage, thus unfit for quality control in large production.

The purpose of the invention is to provide a technical method that is used to produce rAAV conveniently and largely and a full-function helper virus HSV-rc. The purpose of the invention is carried out by providing recombinant cosmid that contain rep-cap genes and the construction method thereof, HSV-rc and the construction method thereof as well as the method of producing rAAV by using HSV-rc.

The full-function helper virus HSV-rc presented in the invention is a recombinant HSV-1 virus with the characteristic of a copy of rep-cap genes (4.3KB, the direction is not restricted) being inserted into the genome of HSV-1. In the two HSV-rc virus constructed by the invention, rep-cap genes are inserted respectively into the site Xbalin UL2 gene (encodes uracil-DNA glycosylase) and UL44 gene(encodes glycoprotein C) of HSV-1, and the obtained recombinant viruses are named respectively HSV-rc/ΔUL2 (figure 1a) and HSV-rc/ΔUL4 (figure 1b). The products encoded by UL2 and UL44 genes are not necessary for the proliferation and passage of HSV-1 in in-vitro cell culture. Both of the two recombinant HSV1 viruses may be proliferated and subcultured stably in HSV sensitive cells (e.g., BHK-21).

The HSV-rc virus generated by the invention is produced based on the modification for a set of cosmids (Set C cosmid, including cos6, cos14, cos28, cos48, cos56) (Conningham, C Davision AJ. A cosmid-based system for constructing mutants of herpes simplex virus type 1. Virology, 1993, 197:116-124) that contain the full-length genome of rAAV virus particle. Firstly, the rep-cap genes of AAV-2 are inserted into the genome of HSV-1 contained in one of the cosmids by rcombinant DNA technology.

Then the recombinant cosmid that has been inserted rep-cap genes is cut with the other four corresponding cosmids together by enzyme to remove the backbone part of the cosmids before being co-transfected into HSV1 sensitive cells such as BHK-21 using liposome method. Recombinant virus is generated through homologous recombination of the five fragments of HSV-1.

The cells transfected with rAAV vector plasmid that contains reporter gene GFP (Green fluorescence protein) are infected by the recombinant HSV1 virus, and the obtained supernatant of the cell lysate is used in infecting the cultured mammalian cells, a lot of green cells can be observed under fluorescent microscope (the wavelength eradiated is 490nm). The result demonstrates that the rAAV viruses generated are infectious, by which exogenous genes can be introduced into cells for expression.

The recombinant plasmid pEBUF5 that contains GFP gene is constructed based on the plasmid pBDZ(+) (Chinese patent application 97116981.0) in the invention. The plasmid is introduced into 293c18 cells (ATCC CRL10852, F9766, the gene EBNA1 of EBV is contained and expressed therein), and the resistant cell strain HygromycinB thus obtained is named 293c18/EBUF5. A lot of infectious rAAV/GFP virus particles may be produced conveniently by using HSV1-rc virus to infect the cell strain 293c18/EBUF5 that carries stably the rAAV vector plasmid pEBUF5, thus a large-scale production of rAAV can be carried out by this method.

The original biological materials that are used in the invention to construct recombinant HSV-rc are:

Set C cosmids: consist of five cosmids that carry respectively the full-length genome of HSV-1: cos6, cos14, cos28, cos48, cos56 (Conningham C, Davision AJ. A cosmid-based system for constructing mutants of herpes simplex virus type 1. Virology, 1993, 197:116-124), and is kindly presented by Davision AJ. There are terminal repeat sequences between each terminal HSV-1 fragment loaded in one cosmid and that in another cosmid, which is the basis of homologous recombination for the five genome fragment of HSV-1 in cells.

pSub201: Samulski et al, A recombinant plasmid from which an infectious adeno-associated virus genome can be excited in vitro and its use to study viral replication.

The production method of recombinant virus HSV-rc

The same strategy and method as producing recombinant virus HSV1-lacZ100 (Wu, XiaoBing et al, Chinese patent application number 98101753.3) are used.

In the HSV-1 genome fragments that are loaded in cos6 and cos56 cosmids there is respectively one single enzyme cleavage site Xbal that are located respectively in UL2 and UL44 genes. Rep-cap genes are removed from pSub201 by cutting with Xbal, and inserted into the site Xbal in cos6 and cos56 to construct recombinant cosmids cos6-rcΔUL2 (figure 2a) and cos6-rcΔUL44(figure 2b). The two recombinant cosmids are preserved in the strain of E. coli DH5α (MAX Efficiancy DH5α, GIBCO#18258-012). The strains that respectively contain the two recombinant cosmids have been

deposited in China General Mcirobiological Culture Collection Center on September 24, 1998. The accession numbers are respectively CGMCC No.0361-1 and CGMCC No.0361-2.

cos6-rcΔUL2 and cos14, cos28,cos48,cos56 together is called Set H (figure 2a), and cos56- rc ΔUL44 and cos6, cos14, cos28, cos48 together is called Set I (figure 2b). The five cosmids of Set H or Set I are mixed in equal mol, cut by PacI to remove the backbone of cos, and co-transfected into BHK-21 cells by liposome, homologous recombination occurs among the five HSV-1 fragments in cells and the recombinant virus HSV-rc is generated: the cells appear pathological 5 days later, and the cultured supernatant is collected after complete pathological, and centrifuged at 2000r/min for 5min, and the obtained supernatant is sotred at –20°C. The recombinant HSV-rc produced by Set H is named HSV1-rcΔUL2 (figure 1a), and the recombinant HSV-rc produced by Set I is named HSV1-rcΔUL44 (figure 1b). The probability of producing recombinant HSV-1 that contains target DNA fragment through the method is 50-100%. Pure recombinant virus can be easily obtained through plaque screen.

The preparation of the full-function helper virus used for the producing rAAV and the use thereof of the invention are specified in the following examples, which should not be construed as limitations for the contents of the invention.

EXAMPLE 1 the preparation of cosmid DNA

Cosmid DNA were extracted by the method of alkaline lysis according to Molecular Cloning –A Laboratory Manual, 2nd edition (Sambrook J. et al, 1986), and purified by the method of polyethylene glycol precipitation.

EXAMPLE 2 the preparation of recombinant HSV-rc

The five cosmids of Set H or Set I were mixed respectively in equal mol, and cut by PacI to remove the backbone of cos (separation removal was not necessary), and extracted respectively one time by phenol, phenol/chloroform (1:1), the supernatant was removed, and the DNA therein was precipitated with 2.5 times absolute ethanol. 20ul Lipofactamine (GIBCO BRL) and 10ug DNA were co-transfected according to the product description into 80% confluenced BHK-21 cells (about 2×10^6 cells), homologous recombination would occur among the five HSV-1 fragments in cells and the recombinant virus HSV-rc would be generated. The cells were cultured at 37□ in 1640 medium supplemented with 2% FBS after transfecting for 24h, and the medium was changed one time per day. The cells appeared pathological 5 days later, and the cultured supernatant was collected after complete pathological, and centrifuged at 2000r/min for 5min, and the obtained supernatant is stored at –20°C. The recombinant HSV-rc produced by Set H is named HSV1-rcΔUL2, and the recombinant HSV-rc produced by Set I is named HSV1-rcΔUL44. Pure HSV1-rcΔUL2 and HSV1-rcΔUL44 could be obtained after two times plaque purity of the recombinant viruses.

EXAMPLE 3 the construction of cell strain 293c18/pEBUF5

The recombinant plasmid pEBUF5 that contained GFP gene was constructed based on the plasmid pBDZ(+) (Chinese patent

application 97116981.0), the structure of pEBUF5 was shown in figure 3. The plasmid pEBUF5 was introduced into 293c18 cells (ATCC CRL 10852, F9766) by the method of liposome, and the cells were cultured with 200ug/ml Hygromycin B selection for 10-15d, the obtained resistant cell strain was named 293c18/EBUF5.

EXAMPLE 4 the cells having been transfected with pAAV-GFP were infected by HSV-rc to prepare rAAV-GFP

The cells transfected with rAAV vector plasmid that contained reporter gene GFP (Green fluorescence Protein gene) were infected by recombinant HSV1, the transfected cells were froze and thawed for 4 cycles to release rAAV-GFP after pathological, the cell debris were removed by low centrifugation, and the supernatant was inactivated at 56°C for 30min for use in infecting cultured mammalian cells.

EXAMPLE 6 the cell strain 293c18/pEBUF15 was infected by HSV-rc to prepare rAAV-GFP

The cell strain 293c18/pEBUF15 that carried rAAV vector plasmid pEBUF5 stably was infected by 0.5-5moi HSV1-rc, the cells appeared complete pathological 24-28h later, the cells and the medium thereof were froze and thawed for 4 cycles, and centrifuged at 1000r/min for 5min, a lot of rAAV-GFP virus were contained in the supernatant. Lots of infectious rAAV/GFP virus particles may be produced conveniently by this method, thus large-scale production of rAAV could be carried out.

EXAMPLE 7 rAAV viruses were transduced into cultured cells

1ml supernatant of rAAV-GFP viruses were added into the cultured BHK cells (with 80% confluence), a lot of green cells could be observed under fluorescent microscope (the wavelength eradiated is 490nm) 24-28h later. The result demonstrated that the generated rAAV viruses were infectious, by which exogenous genes could be introduced into cells for expression.

Figure 1a illustration of genome structure of recombinant HSV1-rc/ΔUL2
Figure 1b illustration of genome structure of recombinant HSV1-rc/ΔUL44
Figure 2a structure of cos6-rc/ΔUL2 and the cosmid Set H combinations
Figure 2b structure of cos56-rc/ΔUL44 and the cosmid Set I combinations
Figure 3 structural illustration of pEBUF5

What we claimed is:

The present invention relates to the field of genetic engineering in virus, particularly to the replicating and packaging function system that are necessary for large production of recombinant

adeno-associated virus (rAAV). The invention relates to a method of producing recombinant herpes simplex virus (HSV1-rc) that is loaded with rep-cap genes (4.3kb) of type 2 adeno-associated virus (AAV-2) and the use thereof in the production of recombinant adeno-associated virus.

1. A strategy of the invention for preparing a helper virus HSV-rc that can provide the full-function needed for the replication and package of rAAV, with the characteristics that, gene manipulation is done on a set of cosmids (Set C) that contain the full-length genome of HSV-1, rep-cap genes are inserted into the genome fragment of HSV-1, the resulting cosmid is co-transfected with the other four cosmids into cells, and the recombinant HSV-1 that contains rep-cap genes is obtained. The recombinant virus can provide both helper virus function needed for producing rAAV and the function of rep-cap genes, and thus is a full-function helper virus for producing rAAV.

2. Two HSV-rc constructed in the invention with that characteristics that, rep-cap genes are inserted respectively into the XbaI site in UL2 gene (encodes uracil-DNA glycosylase) and UL44 genes (encodes glycoprotein C) of HSV-1, and the obtained recombinant viruses are called respectively HSV-rc/ΔUL2 and HSV-rc/ΔUL4.

3. Process for inserting rep-cap genes into the HSV-1 genome fragments loaded in cosmids. Taking advantage of the characteristic of one single enzyme cleavage site XbaI (locates respectively in UL2 and UL44 genes) existing respectively in the HSV-1genome fragment loaded in cos6 and cos56 cosmids, rep-cap genes are removed from pSub201 by cutting with XbaI, and inserted into the site XbaI in cos6 and cos56 to construct recombinant cosmids cos6-rcΔUL2 and cos6-rcΔUL44. The two recombinant cosmids are preserved in the strain of E. coli DH5α (MAX Efficiency DH5α, GIBCO#18258-012). The strains that respectively contain the two recombinant cosmids have been deposited in China General Mcirobiological Culture Collection Center on 24th September 1998. The accession numbers are respectively CGMCC No.0361-1 and CGMCC No.0361-2.

4. The strategy, HSV-rc, or process according to claims 1, 2, or 3, wherein rep-cap genes are inserted into the other parts of the genome of HSV-1 through gene manipulation on a set of cosmids that contain full-length genome of HSV-1, and the recombinant HSV-rc with different inserting positions are produced.

5. The strategy, HSV-rc, or process according to claims 1, 2, or 3, wherein the recombinant HSV-rc with rep-cap genes being inserted into more than two positions.

6. The method of producing rAAV by infecting the cell strain that contains rAAV vector element using the recombinant virus HSV-rc according to the present invention.

ABSTRACTS

The present invention includes a method of producing recombinant herpes simplex virus (HSV1-rc) that is loaded with rep-cap genes of type 2 adeno-associated virus (AAV-2) and the usage thereof in the production of recombinant adeno-associated virus (rAAV). The recombinant herpes simplex virus can provide the complete helper functions that are necessary for rAAV plasmid to be replicated and packaged to be rAAV virus particles, and can be used in large preparation of rAAV. The production of HSV1-rc is carried out based on the modification for a set of cosmids (Set C cosmid, including cos6, cos14, cos28, cos48, cos56) that contain the full-length genome of rAAV virus particle. Firstly the rep-cap genes of AAV-2 are inserted into the genome of HSV-1 in one of the cosmids by recombinant DNA technology, for example inserted into the HSV1UL2 gene in cosmid 6 to construct cos6-rcΔUL2, or inserted into the HSV1UL44 gene in cosmid 56 to construct cos56-rcΔUL2. Then the recombinant cosmid that has been inserted rep-cap genes is cut with the other four corresponding cosmids together by enzyme to remove the backbone part of the cosmids, and transfected into HSV1 sensitive cells such as BHK-21 by the method of liposome. HSV1–rc is generated through homologous recombination among the five fragments of HSV-1 in the cells. Lots of infectious rAAV virus particles can be produced by using HSV1–rc to infect the cells transfected with rAAV vector plasmid or the cell strain that carry stably rAAV vector plasmid. Exogenous genes can be introduced into mammalian cells for expression by using rAAV thus produced.